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29 April 1981

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The purpose of this contract is to support pilot studies into the possibility of utilizing various biological molecules obtained as natural products which can control the immunologic aspects of inflammation (i.e. inhibit lymphocyte function) and thereby possibly prolong the survival of organ grafts after transplantation.

This contract is divided, then, into three sub-projects each of which will be summarized succintly, but separately.

1. Lymphocyte chalone.

For close to two decades it has been postulated that there exist naturally-occurring materials within cells which inhbit specifically the mitotic activity of those cells. These specific endogenous mitotic inhibitors have been given the name "chalone" from a Greek nautical expression meaning "to slack off" the mainsail and slow down. No chalones have, as yet, been unequivocally purified.

We have approached this project and problem by preparing aqueous extracts of fresh calf thymus and isolating the various inhibiting fractions therin of two-way Mixed Lymphocyte Culture produced transformation of lymphocytes in vitro. The assay involves the use of splenic lymphocytes prepared from inbred mice with a histoincompatible loci on their lymphocytes (BALB 6 and C57 BL). These lymphocytes will, in Microwell plates, each transform the other, thus producing large numbers of large vascularized lymphoblasts which can be identified morphologically in tissue culture microscopes (even by biochemists!) and after 72 hours, quantitative determination of this transformation can be made by measuring the six hour incorporation of radioactive thymidine into these cells.

Aqueous extracts of thymus were resolved on ultragel G-200 Sephadex columns into large, middle and small molecular weight components. The large molecular weight material was found to be capable of inhibiting lymphocyte transformation by a cytotoxic process leading to the discovery of Houck's law, namely "Dead cells don't divide". The middle size molecules (molecular weight 40,000 to 60,000 daltons) turned out to apparently inhibit thymidine uptake but did not inhibit lymphoblast transformation morphologically, by its activity on thymidine (i.e. it was a thymidine kinase rendering the label impossible of entrance into the transforming cell) and the smaller molecular weight material contained inhibition of MLC transformation which appeared neither cytotoxic nor due to thymidine kinase activity.

The small molecular weight material, in a range from 10,000 daltons down, was further subdivided on G-25 exclusion column chromatography and 85% of the activity was found at a molecular weight equivalent to bacitracin (i.e. 1400 daltons). Bleomycin and streptomycin sulfate were also standards which indicated the molecular weight of 1400 daltons for the inhibitor. This material was isolated by preparative electrophoresis and identified ultimately as spermine. This polyamine will inhibit lymphocytes at a few micrograms/ml in the presence of fetal cal serum. The calf serum contains an enzyme (polyamine oxidase) which presumably converts the spermine to an aldehyde which, in turn, is capable of inhibiting lymphocyte transformation perhaps five times more effectively on a dose basis than it inhibits the proliferation of fibroblasts in vitro.

A careful perusal of the literature of chalones indicates that a very large number of inhibitory principles could be spermine, either traveling on an anionic polyelectrolyte, like transfer RNA, or on other anionic macromolecules. Spermine has a molecular weight of 200, but because of its very high cationic quality, it binds a sufficiently large amount of water to make a large hole in the solvent which would cause it to be excluded from Sephadex or Biogel with a spuriously high molecular weight of 1400 daltons. Most of the lymphocyte chalone reports in the literature are due largely to spermine. The proof of this is simply to run the two-way MLC studies not in fetal calf serum, but in human serum, which does not contain significant amounts of polyamine oxidase. It takes over 50 times as much spermine to inhibit MLC transformation in human serum than in fetal calf serum.

Careful study using G-25 columns of the material held way back in a small molecular weight area on Ultragel of thymic extracts indicated that another 10% of the inhibitory capacity of this extract was due to cold thymidine nucleotides diluting out the pool size of the thymidine label. The remaining 5% of the inhibitory activity was neither nucleotide nor polyamine and was investigated in the hope that this would prove to be the elusive chalone. The assays of this inhibitory activity were done in human serum.

The final procedure for the isolation of this latter material is as follows: ammonium acetate (50 mmol) extracts of fresh thymus were prepared, and after clarification by centrifugation, precipitated with 60% ethanol. The supernatant from this precipitate was then mixed with large volumes of acetone and a precipitate produced. All of the inhibitor described above was found in this acetate precipitate.

This precipitate was reconstituted in ammonium acetate and subjected to ion-exchange chromatography using either cationic exchanger AG-50 or anionic exchanger AG-1. All of the inhibitory activity passed effectively through the cationic exchanger indicating that the inhibitor was either anionic or neutral in charge and all of it was retained on the anionic inhibitor suggesting that it must, in fact, be an anionic molecule. This material elutes appropriate standardized G-25 column at a molecular weight of 600 daltons.

The inhibitory activity is destroyed by 4N hydrochloric acid hydrolysis (unlike polyamine) and by acetylation indicating a free amino group despite its anionic nature.

This material can be further purified by elution from LH-20 which completes its desalting and produces a peptide fraction which at 20 μg/ml will inhibit by 50% the thymidine uptake of two-way MLC. This fraction, which is still impure, does not inhibit the thymidine uptake of K562 erythroblastoid cells, thus indicating a considerable degree of specificity even at a still impure state.

We are currently investigating the inhibitory capacity of this fraction against B cells (mouse lymphocytes transformed by LPS) and are attempting to further purify it by silisic acid and ultimately HPLC.

2. Bacterial Inhibitor

We have cultivated a pure culture of Pseudomonas recovered from a patient with a remarkable tolerance for cadaver kidney graft. We have isolated from within this bacterium a protein with a molecular weight of perhaps 80,000 daltons which apparently paralyzes both human and mouse lymphocytes in vitro.

This material is prepared by disrupting the harvested Pseudomonas aeruginosa cells by shaking with glass beads at high speed and clarified with streptomycin sulfate to remove the nucleic acids, leaving a clear supernatant. This supernatant is precipitated with 30% alcohol to remove some extraneous components and all of the inhibitory activity is concentrated at 60% precipitate with ethanol. This precipitate is re-dissolved in saline and chromatographed on Ultragel 200, and separated from various proteases which can destroy it. The 80,000 dalton sized portions of the eluate are, in turn, concentrated and subjected to Red Dye Ligand Chromatography. All of the inhibitory activity is eluted at the beginning of the salt gradient from this column and while heterogeneous, still represents highly purified inhibitor fraction.

Preliminary experiments in mice indicate that this material has lost most of its toxicity and, when administered every other day at a dose of 0.1 or 1 mg/mouse, will significantly prolong the survival of C-57 skin transplant on BALB-C mice. Specifically, the normal time required to reject the skin grafts is 10 to 12 days. We can prolong this up to 15 days with the bacterial product at the moment. We are in the process of making more of this material and trying to complete its purification while in more detailed experiments attempting to demonstrate its activity biologically as an immunosuppressive agent.

3. Poly-polyamine.

Most recently we have been attempting to develop a series of cross-linked polymers of spermine to study the ability of these increasing-sized polymers to inhibit lymphocyte transformation in vitro. We have prepared a dimer of spermine by cross-linking it with gluteraldehyde and separating the molecule product as a dimer by exclusion column chromatography. Spermine elutes on Sephadex with a molecular weight of apparently 1400 daltons or 7 times its true mass. The dimer, not surprisingly, has an apparent molecular weight of 3500 daltons. Five µg/ml will inhibit lymphcyte transformation in human serum. This is the same activity displayed in fetal calf serum so obviously this dimer does not require or react with polyamine oxidase.

We are going to attempt to develop further the polymerization of polyamine by isolating dimers which will then be further cross-linked with glutaraldehyde. Theoretically, we would hope this might become an increasingly effective molecule for the inhibition of lymphocyte function both in vitro and in vivo.

Preliminary studies have indicated that the polyamine, when dimerized, will, in fact, delay skin graft rejection across histoincompatible mice (as described above) from 12 to 14 days. This is borderline significance, but then the injection of 100 µg of the material per mouse every other day may not be the ideal dose, route or compound (i.e. the tetramer might be better). We hope to explore this during the coming year.

Summary:

We have been exploring the efficacy of three different types of compounds capable of inhibiting lymphocyte function in vitro in terms of their potential application to control inflammation and particularly, organ graft rejection in vivo. Three papers have been published describing the results of this work to date:

a) Patt, L.M. and J.C. Houck. The incredible shrinking chalone. Febs. Lett. 120(2): 163-170, Nov. 1980.

b) Patt, L.M., J.M. Gleisner, D.M. Barrantes and J.C. Houck. Low molecular weight inhibitors of lymphocyte transformation. (In press, Pharmacology).

c) Gleisner, J.M., L.M. Patt, C.A. Ramthun and J.C. Houck. Pulmonary polyamine permeability factor. Inflammation (In Press).

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